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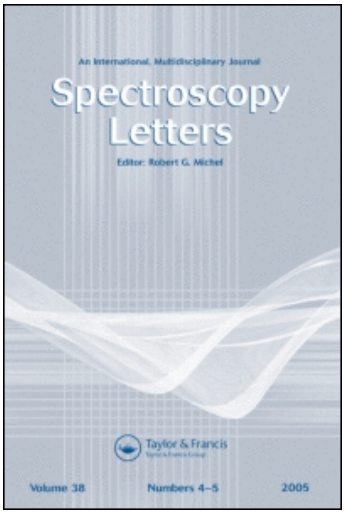

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Variations in the Concentrations of Cu and Zn and in the Ratio of Cu to Zn in Whole Blood and Hair Samples from Hepatocellular Carcinoma Patients and from Healthy Controls in Taiwan

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Variations in the Concentrations of Cu and Zn and in the Ratio of Cu to Zn in Whole Blood and Hair Samples from Hepatocellular Carcinoma Patients and from Healthy Controls in Taiwan

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ABSTRACT An atomic absorption spectrophotometer was used to determine concentrations of copper and zinc and the ratio of Cu to Zn in samples of whole blood and hair from hepatocellular carcinoma (HCC) patients ($n = 51$) and from healthy controls ($n = 50$) in Taiwan. Our results indicate that the HCC patients have higher copper concentrations and higher ratios of Cu to Zn than do the healthy controls both in whole blood and hair samples, but only the concentration of copper and the ratio of Cu to Zn in whole blood were significantly different ($p < 0.001$ and $p < 0.05$). Conversely, a lower concentration of zinc was found in whole blood and hair samples of HCC patients. Similarly, only the concentration of zinc in whole blood showed a significant difference ($p < 0.001$). We concluded that the whole blood concentrations of copper and zinc and the ratio of Cu to Zn seemed to have a higher correlation with HCC. Thus, we suggest that a sample of whole blood may be a more suitable diagnostic sample than is a hair sample for HCC.

KEYWORDS copper, hair, hepatocellular carcinoma, whole blood, zinc

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INTRODUCTION

Hepatocellular carcinoma (HCC) has become one of the top three causes of death in Taiwan.[1] Worldwide, HCC is estimated to cause between 250,000 and 1 million deaths annually.[2] To improve the prognosis of HCC, it is important to diagnose its presence as early as possible. Prescreening for HCC in an early stage is widely believed to be an effective form of prevention of HCC. Several diagnostic procedures, including computed tomography, magnetic resonance imaging, and biochemical signals have been used, but they have proved to be

less than satisfactory in lowering spending on large-scale prescreening for HCC.[3–6]

The biochemical and nutritional roles of copper and zinc are widely recognized in the human body, and copper and zinc are constituents of many metalloproteins and metalloenzymes in normal metabolism.[7] Trace element contents and their ratios are frequently reported to be good biomarkers for diagnosis of various cancers.[8–11] Previously, we have reported higher whole blood concentrations of calcium, copper, and iron and lower selenium and zinc levels in HCC patients.[1] Nevertheless, the diagnostic value of the serum concentrations of copper and zinc and the ratio of Cu to Zn in HCC have been reported frequently.[8,12–15] In contrast, the diagnostic value of hair concentrations of copper and zinc and the ratio of Cu to Zn in the HCC have seldom been reported. There have been no documented reports for the determination of the ratio of Cu to Zn in whole blood and hair samples of HCC patients and healthy controls simultaneously. Thus, we not only compare the diagnostic value of these measurements in HCC patients and healthy controls in whole blood and hair samples simultaneously but also try to suggest a more useful diagnostic sample in HCC prognosis with the atomic absorption spectrophotometry (AAS) method.

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MATERIALS AND METHODS

Confirmation of Conditions of HCC

Dr. King-Jen Chang was responsible for confirming the condition of HCC. In this study, 51 male HCC patients of age distribution 55.5 ± 13.0 years were enrolled. All these patients had been diagnosed with HCC at the National Taiwan University School of Medicine. The healthy controls were individuals who underwent an ordinary health examination in the same hospital. They were randomly selected and had no record of any cancer or liver complications. The controls included 50 males with an age distribution of 58.1 ± 14.3 years.

Sampling and Pretreatment of Samples

Sampling

An authoritative surgical doctor of the Department of Surgery at the National Taiwan University School of Medicine collected all samples. In obtaining samples of whole blood and hair at one time from each HCC patient and healthy control, it is necessary to consider the difference of copper and zinc concentrations in samples caused by various factors such as sex, age, nutritional condition, genetic factors, body mass indexes, and living conditions.[1,9] Stringent screening and control are required in sample collection so as not to affect the reliability of the results of the analyses.

The hair samples were collected from the nape section of each HCC patient and each healthy control. Hair samples were cut near the scalp area with thin-blade stainless steel scissors. The length of hair samples ranged from 1.0 to 3.0 cm. Hair samples were accurately weighed to 1.000 ± 0.200 g, placed inside polyethylene bags, and stored in a controlled environment at 25°C and 65% relative humidity.[5] After the hair sampling, a 10.0-mL whole blood sample was also taken from the same HCC patients and healthy controls and immediately packed in a polyethylene plastic tube. All samples were preserved in a freezer at −18°C before being digested and analyzed within 1 week.[1]

Pretreatment of Hair Samples

Hair samples were immersed in a 65-mL mixture of normal hexane, ethyl alcohol, and acetone (4:2:1 v/v) two times. Each immersion lasted 1.5 h. Then, the samples were rinsed under deionized distilled water four times and immersed in 65 mL acetone for 15 min. The samples were given a final rinse under deionized distilled water three times, filtered with paper, dried at ambient temperature, and prepared for the digestion procedure.[9]

Sample Digestion

Whole Blood Digestion

Digestion of whole blood samples was carried out as follows: Defrost 10 mL whole blood sample at room temperature of 20°C and pour into 250-mL vessel. Add 20-mL 65% nitric acid and heat in a microwave at 15% power for 15 min and then at 25% power for 10 min. Add 9 mL 97% sulfuric acid and 8 mL 60% perchloric acid and heat in a microwave at 30% power for 10 min and then at 40% power for 35 min. Finally, add 10 mL deionized distilled water and heat

at 40% power for another 8 min. The digest solutions were diluted to 50 mL with highly purified deionized distilled water. Aliquots then were taken for atomic absorption spectrophotometer analysis.[1]

Hair Digestion

A hair sample was weighed (0.200 ± 0.100 g) and then placed inside a 250-mL microwave digester vessel. Nitric acid (10 mL) was added followed by heating in a microwave using less than 30% power for 5 min. Then, 10 mL deionized distilled water was added followed by 40% power heating for 25 min and 0% power heating for 10 min. Finally, 2 mL hydrogen dioxide (H_2O_2) was added, followed by 65% power heating for 5 min. After the heating procedures, vessels were taken out under normal pressure and temperature. All digested solutions were diluted to specific volumes with deionized distilled water for atomic absorption spectrophotometer determination.[9]

Apparatus and Reagents

Suprapure-grade reagents purchased from Merck (Merck Corp., Darmstadt, Germany) and highly purified water (18 MΩ) were used. Stock solutions contained 1000 mg/L copper and zinc. Working standard solutions were prepared from Merck Titrisol standards by dilution with highly purified deionized water. Containers made of quartz, Teflon, or polypropylene were used throughout. They were immersed in 8 N HNO_3 overnight and washed with several changes of deionized water prior to use.[1,9]

Analysis Methods

Prolab Max Digester (Prolab Corp., Paris, France) was used for whole blood digestion[1] and CEM-MD2000 microwave digester (CEM Corp., Matthews, NC, USA) was used for hair digestion.[9] An atomic absorption spectrophotometer model Z-8200, (Hitachi Corp., Tokyo, Japan) was used, and flame AAS was used for copper and zinc analysis.[1,9]

Quality Assurance and Quality Control

Because of the diversity of samples, the recovery and coefficient of variation (CV%) of standard reference materials were used to validate the accuracy and precision of the measurement of copper and zinc. Standard reference materials were used to establish comprehensive quality assurance and quality control in the laboratory. We used NIST (U.S. National Institute of Standards and Technology) SRM (Standard Reference Material) 1598 serum as standard (Beltsville Human Nutrition Research Center, U.S. Department of Agriculture, Beltsville, MD, USA) for whole blood analysis and NIES CRM No. 13 (National Institute for Environmental Studies Certified Reference Material No. 13 [human hair]; Tokyo, Japan) for hair analysis.

Accuracy and Precision

To ensure the accuracy and the precision of analytical data, standard reference materials, and a comprehensive quality assurance and quality control system in the laboratory are necessary. We used NIST SRM 1598 serum standard reference material and standard material NIES CRM No. 13 (human hair) as standards. The recovery rate and CV% were used as a basis for quality assurance to ensure the accuracy and precision of analytical data. A series of standard solutions containing the following concentrations of copper and zinc ions were prepared using deionized distilled water and stock solutions (1000 ppm): 0.00, 0.10, 0.20, 0.40, 1.00, and 2.00 μg/mL. The linear regression coefficient of the standard calibration curve for each element was greater than 0.9995.

Our results showed that the overall mean recovery for whole blood and hair concentrations of copper and zinc were greater than 97.5%. Meanwhile, the CV% of whole blood and hair concentrations of copper and zinc were less than 6.8%. Therefore, we conclude that our method is applicable to the analysis of copper and zinc concentrations both in whole blood and hair samples. The details of NIST SRM 1598 serum standard reference material and NIES CRM No. 13 (human hair) certified reference material recovery and CV% are given in Table 1 and Table 2.

Statistical Analysis

In this study, for conceptual simplicity and ease of comparison parametric analysis, the changes in the clinical findings of the groups were tested for the level of significance by two-simple *t*-test analysis. Means between two groups (HCC patients and healthy controls) were subjected to two-sides analysis of variance and compared by use of the Gosset range

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TABLE 1 **The Recovery and Coefficient of Variation of Copper and Zinc in the Standard Material NIST SRM 1598 Serum**

Element	Certified value (μg/mL)	CV (%)	Analyzed value (μg/mL)	CV (%)	Recovery (%)
Copper	0.72 ± 0.04	5.5	0.71 ± 0.04	5.6	99.5
Zinc	0.89 ± 0.06	6.7	0.86 ± 0.06	6.8	97.5

Each value is the mean ± SD of 3 runs.

SD, standard deviation; CV%, coefficient of variation; NIST SRM 1598, serum National Institute of Standards and Technology Standard Reference Material 1598 serum.

(Student's *t*-test range). A statistical graphics package STATISTICA (version 6.0, StatSoft, Inc, Tulsa, OK, USA) was used to complete the computation of various statistical data. If the p value for the mean concentrations of copper and zinc and the ratio of Cu to Zn between any two groups is less than 0.05 ($p < 0.05$), then the difference will be significant. Values were expressed as means ± SD.

All of the subjects provided their informed consent as approved by the college medical ethics committee. Also, this study was approved by the Committee of Research and Development at St. John's University (Taipei, Taiwan).

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RESULTS

Variations in the Concentrations of Copper and Zinc in Whole Blood and Hair Samples from the HCC Patients and from the Healthy Controls

In this work, higher concentrations of copper in the HCC patients was found not only in whole blood samples (1.74 ± 0.36 μg/mL > 1.31 ± 0.30 μg/mL; $p < 0.001$), but also in hair samples (10.95 ± 4.52 μg/g > 10.91 ± 7.05 μg/g; $p > 0.05$). Only the whole blood sample had a significant difference. Conversely, lower concentrations of zinc were found in the HCC patients both in the whole blood samples (10.67 ± 2.67 μg/mL < 19.04 ± 10.37 μg/mL; $p < 0.001$) and in hair samples (154.8 ± 51.6 μg/g < 166.0 ± 74.6 μg/g; $p > 0.05$). Similarly, only the whole blood sample of zinc had a significant difference. The details of the concentrations of copper and zinc in whole blood and hair samples for the HCC patients and the healthy controls including their distribution are shown in Table 3 (for whole blood samples) and Table 4 (for hair samples) respectively.

Variations in the Ratio of Cu to Zn in Whole Blood and Hair Samples from the HCC Patients and from the Healthy Controls

With regard to Table 5, our findings demonstrate that the whole blood ratio of Cu to Zn ($0.126 \pm 0.034 > 0.109 \pm 0.042$; $p < 0.05$) and the hair ratio of Cu to Zn ($0.089 \pm 0.065 > 0.087 \pm 0.064$; $p > 0.05$) in HCC patients were both greater than those of healthy controls. Clearly, only the whole blood sample had a significant difference ($p < 0.05$). The details of the ratio of Cu to Zn and their distribution in whole blood samples and in hair samples from the HCC patients and from the healthy controls are shown in Table 5.

TABLE 2 **The Recovery and Coefficient of Variation of Copper and Zinc in the Standard Material NIES CRM No. 13 (Human Hair)**

Element	Certified value (μg/g)	CV (%)	Analyzed value (μg/g)	CV (%)	Recovery (%)
Copper	15.3 ± 1.3	8.4	15.2 ± 0.85	5.5	99.5
Zinc	172 ± 11	6.3	169.4 ± 11.5	6.7	98.5

Each value is the mean ± SD of 3 runs.

SD, standard deviation; CV%, coefficient of variation; NIES CRM No. 13 (human hair), National Institute for Environmental Studies Certified Reference Material No. 13 (human hair).

TABLE 3 **Variation in the Concentrations of Copper and Zinc in Whole Blood Samples of the HCC Patients and the Healthy Controls**

Element	HCC patients (n = 51)	Healthy controls (n = 50)	*t*-test of p value
Copper (μg/mL)	1.74 ± 0.36	1.31 ± 0.30	$p < 0.001$
Range (μg/mL)	2.86∼0.88	2.20∼0.88	
Zinc (μg/mL)	10.67 ± 2.67	19.04 ± 10.37	$p < 0.001$
Range (μg/mL)	21.78∼7.04	55.20∼5.90	

Each value is the mean ± SD of 3 runs.
SD, standard deviation.

TABLE 5 **Variation in the Ratio of Cu to Zn in Whole Blood and Hair Samples of the HCC Patients (n = 51) and the Healthy Controls (n = 50)**

	Ratio of [Cu/Zn] in blood	Ratio of [Cu/Zn] in hair
HCC patients	0.126 ± 0.034*	0.089 ± 0.065
Range of distribution	0.237∼0.073	0.217∼0.020
Healthy controls	0.109 ± 0.042*	0.087 ± 0.064
Range of distribution	0.200∼0.036	0.296∼0.015

Each value is the mean ± SD.
SD, Standard deviation.
*Significantly different than healthy controls ($p < 0.05$).

DISCUSSION

Many reports[8,16–21] have demonstrated that the higher concentration of copper, higher ratio of Cu to Zn, and lower concentration of zinc are commonly found in the serum of HCC patients. Some of these reports[12,13,20] further emphasize that the concentrations of copper and zinc and the ratio of Cu to Zn in serum could be used as a biomarker in the diagnosis of HCC at an early stage. Nevertheless, Nakayama et al.[20] concluded that the mean serum concentrations of copper and zinc and ratio of Cu to Zn could be used in diagnosis for hepatitis disorders including chronic hepatitis and liver cirrhosis. In the current work, we not only indicate that the whole blood concentration of copper and the ratio of Cu to Zn in HCC patients were higher than those of healthy controls but also demonstrate that the whole blood concentrations of zinc in HCC patients were lower than those of healthy controls. Our finding is in agreement with Poo et al.,[8,10] Tashiro et al.,[18] and Nakayama et al.[20] in serum.

Hematology analysis has been commonly used in the determination of trace elements status in the human body,[20–22] but hair analysis has a special advantage in that the contents of trace elements can be backtracked over longer periods of time (3–6 months or even more) and are less affected by the daily diet.[23,24] Furthermore, sampling of hair is easy and harmless for the subjects. Nevertheless, we also indicate the same finding in hair samples as the finding in whole blood samples. We not only indicate the hair concentration of copper and ratio of Cu to Zn in HCC patients were higher than those of healthy controls but also demonstrate that the hair concentrations of zinc in HCC patients were lower than those of healthy controls.

For a better understanding of the diagnostic value of whole blood and hair samples, we further try to compare these two kinds of samples (whole blood and hair samples) using the p value for concentrations of copper and zinc and the ratio of Cu to Zn between HCC patients and healthy controls groups. According to Table 3 and Table 4, samples of the whole blood concentration of copper and zinc show an apparent significant difference ($p < 0.001$) compared with the hair concentration of copper and zinc ($p > 0.05$). The ratio of Cu to Zn in whole blood samples also shows a significant difference ($p < 0.05$) than the ratio of Cu to Zn in hair samples ($p > 0.05$). We conclude that the whole blood sample concentrations of copper and zinc and the ratio of Cu to Zn seem to have a higher correlation with HCC than do the hair sample concentrations of copper and zinc and ratio of Cu to Zn.

The liver (hepatocellular) manufactures a lot of metalloproteins and metalloenzymes to detoxify and regulate normal metabolism in the human body. Zinc plays a role as the most important cofactor in the activation of metalloproteins and metalloenzymes. Lower

TABLE 4 **Variation in the Concentrations of Copper and Zinc in Hair Samples of the HCC Patients and the Healthy Controls**

Element	HCC patients (n = 51)	Healthy controls (n = 50)	*t*-test of p value
Copper (μg/g)	10.95 ± 4.52	10.91 ± 7.05	$p > 0.05$
Range (μg/g)	20.16∼4.71	26.16∼2.49	
Zinc (μg/g)	154.8 ± 51.6	166.0 ± 74.6	$p > 0.05$
Range (μg/g)	304.3∼69.1	265.5∼47.4	

Each value is the mean ± SD of 3 runs.
SD, standard deviation.

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zinc concentrations probably cause less ability for detoxification in the human body. These effects might be attributed to the hepatocellular concentration of zinc as a primary influence on HCC. Some other reports[20,21] conclude that increased concentration of serum copper may be related to liver cirrhosis, chronic hepatitis, even several carcinomas. From these points of view, the serum concentration of copper might be directly attributed to a secondary influence on HCC development. Based on these observations, we suggest that whole blood sample concentrations of copper and zinc and the ratio of Cu to Zn serve as better supportive evidence than do hair sample concentrations of copper and zinc and the ratio of Cu to Zn and as a better biomarker for use in diagnosis of HCC and could act as a secondary prevention method. However, this needs further investigation.

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